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Subversion of the T/B Lineage Decision in the Thymus by Lunatic Fringe-Mediated Inhibition of Notch-1

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Summary

Notch-1 signaling is essential for lymphoid progenitors to undergo T cell commitment, but the mechanism has not been defined. Here we show that thymocytes ectopically expressing Lunatic Fringe, a modifier of Notch-1 signaling, induce lymphoid progenitors to develop into B cells in the thymus. This cell fate switch resulted from Lunatic Fringe-mediated inhibition of Notch-1 function, as revealed by experiments utilizing lymphoid progenitors in which Notch-1 activity was genetically manipulated. These data identify Lunatic Fringe as a potent regulator of Notch-1 during the T/B lineage decision and show that an important function of Notch-1 in T cell commitment is to suppress B cell development in the thymus.

Introduction

T and B cells arise from clonal lymphoid progenitors (LP) (Kondo et al., 1997; Lacaud et al., 1998), but they develop in distinct microenvironments. Hematopoietic stem cells (HSC) generate LP in the bone marrow, where they undergo B cell commitment and maturation in response to inductive signals from stromal cells (reviewed in Akashi et al., 2000a). LP also migrate to the thymus (Wu et al., 1991; Peault et al., 1994), where most T cell development occurs. Thymic LP express the c-Kit receptor tyrosine kinase and are found among CD4/CD8 double-negative (DN) thymocytes. Commitment to the T cell lineage is first evident when $CD44^+$ DN thymocytes express CD25 and high levels of Thy-1 (Zuniga-Pflucker et al., 1995). After T cell commitment, DN thymocytes lose CD25 and CD44 and become CD4/CD8 double-positive (DP) cells that die or choose between the CD4 versus CD8 single-positive (SP) lineages.

Notch proteins are evolutionarily conserved transmembrane receptors that regulate a variety of cell fate decisions in worms, flies, and vertebrates (Artavanis-Tsakonas et al., 1999; Robey, 1999). Recent data have shown that the Notch-1 signaling pathway can influence survival and lineage decisions at multiple stages of T cell development (Robey et al., 1996; Washburn et al., 1997; Deftos et al., 1998, 2000; Kim and Siu, 1998). Moreover, Notch-1 critically regulates the T/B lineage choice. LP from mice lacking Notch-1 (Radtke et al., 1999) or its downstream target HES-1 (Tomita et al., 1999) fail to generate T lineage cells in the thymus, demonstrating that Notch-1 is essential for T cell commitment. Conversely, the expression of a constitutively active Notch-1 mutant (Notch-IC) promotes ectopic T cell development in the bone marrow of athymic mice and inhibits LP from giving rise to B lineage cells (Pui et al., 1999). Thus, Notch-1 activation can induce T cell commitment in a thymus-independent manner, which implies that LP must activate Notch-1 in the thymus but not in the bone marrow to ensure that T cells develop in only the thymus. However, Notch ligands are widely expressed in the bone marrow and thymus (Shawber et al., 1996; Luo et al., 1997; Kaneta et al., 2000; Singh et al., 2000), and it is not yet clear how Notch-1 is regulated in LP to ensure spatially restricted development of the two lymphoid lineages.

The mechanism by which Notch-1 signaling induces T cell commitment has not been defined. Interestingly, Radtke et al. (1999) noted increased numbers of B cells in the thymus of conditional Notch-1 knockout mice and suggested that the absence of Notch-1 signaling may direct thymic LP toward a B cell fate. Alternatively, this abnormality could reflect enhanced migration of peripheral B cells to the thymus and/or augmented intrathymic proliferation of committed B cells. Consistent with the latter possibility is the finding that the thymus contains committed B cell progenitors that can develop into mature B cells when reintroduced into the thymus by intrathymic injection (Mori et al., 1997). Moreover, increased numbers of thymic B cells have been noted in several T cell-deficient strains (Tokoro et al., 1998; Akashi et al., 2000b). Thus, it remains unclear whether failure to activate Notch-1 directs LP toward the B cell fate in the thymus or whether the paucity of T cells in the Notch-1-deficient thymus simply provides more space for committed B cell progenitors to proliferate and differentiate.

Notch signaling can be modified by other proteins, such that only a subset of cells with the potential to respond to Notch signals actually do so. For example, during the development of fly wings and eyes, all cells express Notch and one of the Notch ligands, Delta or Serrate. However, the Fringe protein restricts Notch activation to cells at the dorsal-ventral boundary in these primordial tissues (Irvine and Wieschaus, 1994; Kim et al., 1995; Cho and Choi, 1998; Dominguez and de Celis, 1998). Ectopic expression studies have provided important insights into the mechanism of this restriction by showing that it is the juxtaposition of Fringe-expressing with Fringe-nonexpressing cells that specifies the location of Notch activation at compartment boundaries (Irvine and Wieschaus, 1994; Rodriguez-Esteban et al., 1997). Fringe acts cell autonomously with Notch in developing fly wings (Panin et al., 1997), and recent studies have identified Fringe proteins as Golgi-localized glycosyltransferases that modify Notch receptors (Bruckner et al., 2000; Hicks et al., 2000; Moloney et al., 2000; Munro and Freeman, 2000).

The three vertebrate Fringe proteins, Lunatic, Radical,

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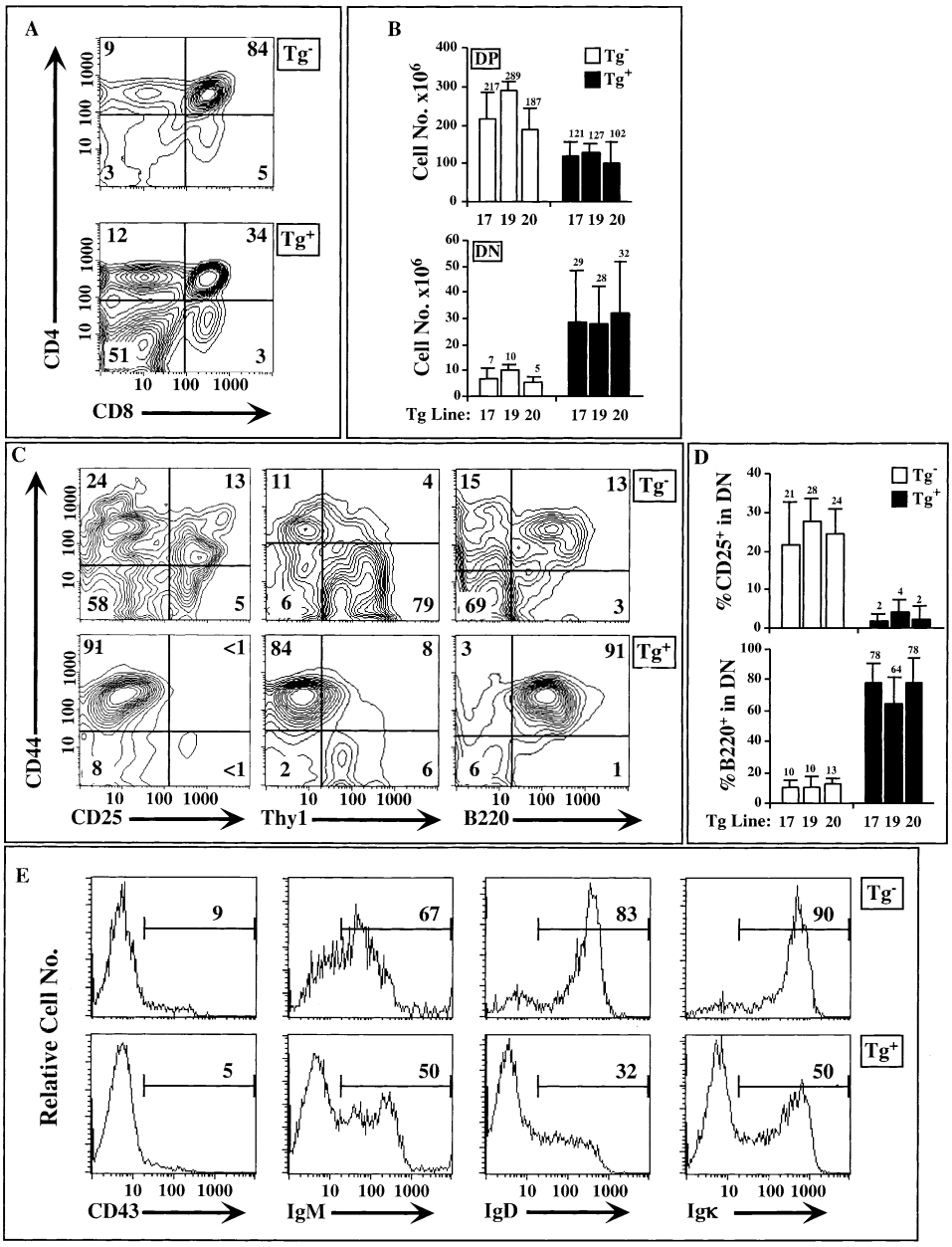

Figure 1. Effect of Ectopic Lunatic Fringe Expression on the Abundance of Thymic T and B Cells

(A) Increased DN and decreased DP thymocytes in Lunatic Fringe transgenic mice. Two-parameter 7% probability contour plots depict surface expression of CD4 versus CD8 on thymocytes from representative 8-week-old Tg^+ versus littermate Tg^- control mice (line 20). The percentages of DN, DP, CD4 SP, and CD8 SP cells in each sample are indicated.

(B) Absolute numbers of DP and DN thymocytes in three different lines of Lunatic Fringe transgenic mice and their Tg^- littermates. The two bar graphs show the absolute numbers (mean ± SD) of DP (top) and DN (bottom) thymocytes in three different lines of Lunatic Fringe transgenic mice (Tg^-, white bars; Tg^+, black bars). The numbers above each bar represent the mean. Mice were analyzed at 3 to 9 weeks of age. The total number of mice analyzed were as follows: line 17, Tg^- n = 9, Tg^+ n = 20; line 19, Tg^- n = 3, Tg^+ n = 7; and line 20, Tg^- n = 9, Tg^+ n = 22.

(C) Predominance of B cells in the DN subset from Tg^+ Lunatic Fringe mice. Two-parameter 7% probability contour plots depict the expression of CD44 versus CD25 (left), CD44 versus Thy-1 (middle), or CD44 versus B220 (right) gated on DN thymocytes from Tg^- (top panels) versus Tg^+ (bottom panels) mice. DN thymocytes were identified by lack of staining with antibodies specific for CD4, CD8, CD3, and TCRβ. The percentages of $CD25^+$ ($CD44^-$ or $CD44^+$), $CD44^-$ Thy-1^+, or $CD44^+$ $B220^+$ cells within the DN subset of each sample are indicated in each quadrant.

(D) The bar graph depicts the percentage (mean ± SD) of $CD25^+$ or $B220^+$ cells within the DN thymocyte subset from Tg^+ mice and their Tg^- littermates for each transgenic line. The mean absolute number of thymic B cells for each transgenic line was 25.7×10^6 (line 17), 24.2×10^6 (line 19), and 28×10^6 (line 20), as compared with $0.7–1.3 \times 10^6$ in their Tg^- littermates (data not shown).

(E) Developmental heterogeneity of thymic B cells from Tg^- and Tg^+ mice. The histograms depict expression of CD43, IgM, IgD, or Igκ on $CD44^+B220^+$ DN thymocytes. The expression of each marker was assessed in thymic B cells from line 20 Tg^+ mice and their Tg^- littermates using four-color flow cytometry to identify $B220^+CD44^+$ thymic B cells within the DN subset. The percentage of $CD44^+B220^+$ cells positive for each marker is shown. We did not observe CD5 expression on thymic B cells Tg^+ or Tg^- mice (data not shown).

and Manic Fringe, are expressed in several tissues where Notch-dependent patterning events occur (Cohen et al., 1997; Johnston et al., 1997). We previously noted (Cohen et al., 1997) that Lunatic Fringe is highly expressed in the thymic medulla, where most mature T cells reside, but not in the cortex, which contains primarily immature cells. To determine whether Lunatic Fringe might modulate Notch-1 activation at the corticomedullary border, we ectopically expressed it in cortical thymocytes under control of the Lck-proximal promoter. Because the Lck-proximal promoter is highly active in T cell-committed immature cortical thymocytes (Shimizu et al., 2001) that also express Notch-1 (Hasserjian et al., 1996), we reasoned that this strategy would be ideal for identifying potential cell-autonomous effects of Lunatic Fringe on Notch-1 activation during postcommitment stages of T cell development. Unexpectedly, we found that ectopic Lunatic Fringe had no effect on the thymocytes themselves. We found instead that transgenic thymocytes act nonautonomously to inhibit Notch-1 activation in LP, thus inhibiting T cell commitment and promoting B cell development in the thymus. These data reveal that a major function of Notch-1 in T cell commitment is to prevent LP from developing into B cells in the thymus. The nonautonomy of Lunatic Fringe function in our system suggests a novel mechanism for Fringe-mediated modulation of Notch signaling.

Results

Ectopic Lunatic Fringe Decreases T Cells and Increases B Cells in the Thymus

In three different lines of Lunatic Fringe transgenic (Tg) mice, we observed a striking increase in the frequency and absolute numbers of DN thymocytes at the expense of DP thymocytes (Figure 1A and 1B). Analyses of the DN subset with other markers revealed a profound decrease (10-fold on average) in the percentage of T cell-committed $CD25^+$ ($CD44^+$ and $CD44^-$) and $CD25^-$ $CD44^-$ DN thymocytes and a concomitant increase in the percentage of $CD25^-$ $CD44^+$ DN thymocytes (Figures 1C and 1D). In striking contrast to Tg^- DN thymocytes, those from Tg^+ mice expressed CD44, but few expressed CD25 or Thy-1 (Figures 1C and 1D), suggesting that they were not committed T cells. These $CD25^-$ $CD44^+$ DN thymocytes were not LP since they did not express c-Kit (data not shown). Rather, up to 94% (64%–78% on average) of transgenic $CD25^-$ $CD44^+$ DN thymocytes expressed the B lineage marker B220 (Figures 1C and 1D). By comparison, only 10%–13% of control DN thymocytes expressed B220, in keeping with previous studies showing that the normal thymus contains few B cells (Akashi et al., 2000b). Moreover, the average absolute number of thymic B cells was $24–28 \times 10^6$ in Tg^+ mice, an increase of up to 200-fold relative to their Tg^- littermates (data not shown).

Most thymic B cells from control mice displayed a $CD43^-$ HSA^{hi} IgM^+ IgD^+ $Ig\kappa^+$ mature phenotype (Figure 1E; data not shown). In contrast, thymic B cells in transgenic mice were more developmentally heterogeneous and included a high frequency (40%–60%) of immature $CD43^-$ HSA^{hi} IgM^- IgD^- $Ig\kappa^-$ pre-B cells, as well as mature $CD43^-$ HSA^{hi} IgM^+ IgD^+ $Ig\kappa^+$ B cells (Figure 1E; data not shown). In fact, the representation of immature and mature thymic B cells in Tg^+ mice closely resembles that in the bone marrow. Collectively, these data show that ectopic Lunatic Fringe inhibits the intrathymic development and/or survival of $CD25^+$ DN and DP thymocytes, but also augments the number of thymic B cells.

Ectopic Lunatic Fringe Induces LP to Adopt the B Cell Fate in the Thymus

To determine whether ectopic Lunatic Fringe acts cell autonomously with Notch-1 in thymocytes to cause the phenotypic alterations we noted above, we constructed mixed bone marrow chimeras. T cell-depleted bone marrow cells from Tg^+ (B6.CD45.2) and/or Tg^- (B6.CD45.1) donors were intravenously injected into lethally irradiated RAG-$2^{-/-}$ (B6.CD45.1) mice. These hosts have an intrinsic defect in antigen receptor gene rearrangement that arrests T and B lymphocyte development. Therefore, all lymphoid cells that have matured beyond the prognenitor stage will be donor derived. Tg^+ bone marrow generated 9- to 30-fold more thymic B cells than Tg^- bone marrow (Tables 1A and 1B), demonstrating that the ability of the Lunatic Fringe transgene to increase the number of thymic B cells is intrinsic to bone marrow-derived cells, as expected. Surprisingly, however, Tg^- donor cells generated high numbers of thymic B cells when coinjected with an equal number of Tg^+ bone marrow cells (Table 1C). In such mixed chimeras, 60%–97% of thymic B cells were derived from Tg^- donor cells (Table 1C; data not shown), indicating that Tg^+ bone marrow-derived cells could nonautonomously augment the number of Tg^- thymic B cells.

Since our donor bone marrow contained B cells, we could not determine whether Tg^+ thymocytes affected B cell migration to the thymus and/or intrathymic B cell proliferation or whether they induced LP to adopt a B cell fate within the thymus. To test the latter possibility, nontransgenic LP from B6.CD45.1 mice were injected directly into the thymus of Tg^+ or Tg^- B6.CD45.2 host mice. Host mice were sublethally irradiated (650 cGy) to facilitate donor cell engraftment while ensuring that some host Tg^+ thymocytes would survive to influence the fate of Tg^- progenitors. Tg^- LP gave rise to DP and SP thymocytes with similar kinetics following injection into Tg^- versus Tg^+ thymic lobes (Figure 2A). However, the absolute number of donor-derived DP thymocytes was 25- to 150-fold less in Tg^+ thymic lobes relative to Tg^- thymic lobes at day 14 postinjection (data not shown) and was 9- to 113-fold fewer by 21 days postinjection (Table 2). Although most LP-derived cells in Tg^- lobes were T cells, small numbers of donor-derived B cells were also evident (Figure 2B and Table 2), in agreement with other studies showing that the normal thymus can support B cell development from LP, albeit inefficiently (Lian et al., 1997).

Strikingly, however, in Tg^+ lobes, 70%–90% of progeny derived from nontransgenic LP developed into B lineage cells by 14 and 21 days postinjection (Figure 2B). This was not simply an increased percentage of thymic B cells, since the absolute number of donor-derived B cells was 3- to 17-fold higher in Tg^+ thymic lobes at 9 and 14 days postinjection (data not shown). By 21 days postinjection, Tg^+ thymic lobes contained

Table 1. Effect of Lunatic Fringe on Thymic B Cell Number Is Intrinsic to Bone Marrow-Derived Cells but Is Cell Nonautonomous

Donor BM	% Donor (Total Thymocytes) CD45.1	% Donor (Total Thymocytes) CD45.2	% B cells in DN	% Donor (DN B cells) CD45.1	% Donor (DN B cells) CD45.2
(A) CD45.1 Tg^-	100	0	1	100	0
	100	0	1	100	0
	100	0	2	100	0
(B) CD45.2 Tg^+	0	100	31	0	100
(Line 20)	0	100	22	0	100
	0	100	18	0	100
(C) CD45.1 Tg^-	63	38	29	94	6
CD45.2 Tg^+	67	33	37	92	8
(Line 20)	56	44	29	91	9
	69	31	29	94	6
	37	64	48	97	3

Bone marrow cells from B6.CD45.1 nontransgenic (CD45.1 Tg^-) or Tg^+ B6.CD45.2 (CD45.2 Tg^+) mice were depleted of $Thy1^+$ T cells using magnetic beads as described. B6.CD45.1.RAG-$2^{-/-}$ host mice were injected intravenously with 10 x 10^6 T cell-depleted bone marrow cells from a single donor (A and B) or with 5 x 10^6 cells from each donor in mixed chimeras (C). Host mice were lethally irradiated (1000 cGy) up to 4 hr prior to injection. Total and $B220^+$ DN thymocytes were evaluated 5 weeks later for expression of CD45.1 or CD45.2, as described for Figure 1. This experiment was performed twice with Line 20 and once with Line 17 with comparable results. Note that CD45.1 thymocytes in chimeric mice could potentially be derived from CD45.1 Tg^- donor or RAG-$2^{-/-}$ host mice. However, as can be seen in (B), $CD45.1^+$ host thymocytes are not detected because the absence of RAG-2 arrests T and B lymphocyte development at the progenitor stage.

2–10 $\times$ 10^6 donor-derived B cells compared with only 2–7 $\times$ 10^5 in Tg^- thymic lobes (Table 2). Examination of other markers expressed by these $B220^+$ progeny showed that most had developed to the $CD43^+$ IgM^- pro-B cell stage by day 9 and progressed to the $CD43^-$ IgM^- stage by day 14 (Figure 2C). Small numbers of mature $CD43^-$ IgM^+ IgD^+ B cells were also evident at day 14 (Figure 2C; data not shown), and donor-derived mature B cells were detected in the spleen and lymph nodes 4–8 weeks later (data not shown).

To verify that transgenic thymocytes could influence the T/B fate of uncommitted progenitors, we conducted similar experiments with purified Lin^- $Sca\text{-}1^+$ $c\text{-}Kit^+$ bone marrow cells from Tg^- donors. This population is highly enriched for HSC (Lin^- $Sca\text{-}1^+$ $c\text{-}Kit^+$ $Thy\text{-}1^{lo}$ IL-7Rα^-) and LP (Lin^- $Sca\text{-}1^+$ $c\text{-}Kit^+$ $Thy\text{-}1^-$ IL-7Rα^+) (Kondo et al., 1997), but lacks common myeloid progenitors (Akashi et al., 2000c). Figure 2D shows that significantly more B cells developed from intrathymically injected HSC/LP in Tg^+ thymic lobes than in Tg^- lobes. By 3 weeks postinjection, approximately 55% of donor-derived thymic B cells had reached the pre-B ($CD43^-$ IgM^-) stage, whereas 10% were immature IgM^+ IgD^- cells, and 35% were mature IgM^+ IgD^+ B cells (Figure 2E). These data demonstrate that ectopic expression of Lunatic Fringe induces LP to commit to the B cell lineage in the thymus. These cells then complete B cell maturation in the thymus following a similar developmental sequence to that seen in the bone marrow.

Ectopic Lunatic Fringe Inhibits Notch-1 Activation in LP

Both ectopic Lunatic Fringe (Figures 1 and 2) and loss of Notch-1 (Radtke et al., 1999) inhibit T cell development in the thymus. Moreover, we show here that LP preferentially adopt the B cell fate in the thymus of Lunatic Fringe transgenic mice (Figure 2). Thus, we hypothesized that ectopic Lunatic Fringe antagonizes Notch-1 activation in thymic LP, thereby inhibiting T cell commitment and promoting intrathymic B cell development. It is well documented that subtle changes in the amount of Notch can have profound influences on cell fate choices (Heitzler and Simpson, 1991; Artavanis-Tsakonas et al., 1999; Robey, 1999). Therefore, we reasoned that if ectopic Lunatic Fringe inhibits Notch-1 activation, LP with a single functional Notch-1 allele ($N1^{+/-}$) (Conlon et al., 1995) should be more susceptible to this inhibition than LP with two functional Notch-1 alleles. To test this hypothesis, Tg^- LP from $N1^{+/-}$ (B6.CD45.1/B6.CD45.2) and $N1^{+/+}$ (B6.CD45.1) mice were mixed at different ratios and injected into Tg^- versus Tg^+ (B6.CD45.2) thymic lobes. Notch-1 gene dosage did not significantly affect the CD4/CD8 profile of thymocytes produced in Tg^- thymic lobes (Figure 3A). However, the frequency of thymic B cells was 2- to 5-fold higher among $N1^{+/-}$ progeny than $N1^{+/+}$ progeny in Tg^- thymic lobes (Figures 3B and 3C). These data suggest that $N1^{+/-}$ LP have an inherently greater potential to adopt a B cell fate in the Tg^- thymus than $N1^{+/+}$ progenitors. Importantly, this inherent B cell potential was dramatically enhanced by ectopic Lunatic Fringe; when $N1^{+/-}$ LP developed in Tg^+ thymic lobes, 58%–78% remained DN (Figure 3A), and typically greater than 80% of the DN cells expressed B220 (Figures 3B and 3C). By comparison, $N1^{+/+}$ LP generated many fewer B cells in the same thymic lobes, and more than 90% were DP and SP thymocytes. These data show that a reduction in Notch-1 gene dosage dramatically potentiates the ability of ectopic Lunatic Fringe to promote B cell development from LP in the thymus.

The ability of Lunatic Fringe to promote preferentially the B cell fate in $N1^{+/-}$ progenitors was also strikingly evident by comparing the ratio of $N1^{+/-}$:$N1^{+/+}$ cells within the B versus T lineages in Tg^- versus Tg^+ thymic lobes (Figure 3D). In the T cell lineage, the $N1^{+/-}$:$N1^{+/+}$ ratio was 2- to 3-fold lower in Tg^+ as compared with Tg^- thymic lobes. In contrast, the $N1^{+/-}$:$N1^{+/+}$ ratio in the B cell lineage was increased 6- to 14-fold in Tg^+ relative to Tg^- thymic lobes. Moreover, in Tg^+ thymic lobes, the ratios of $N1^{+/-}$:$N1^{+/+}$ B cells were 13- to 18-

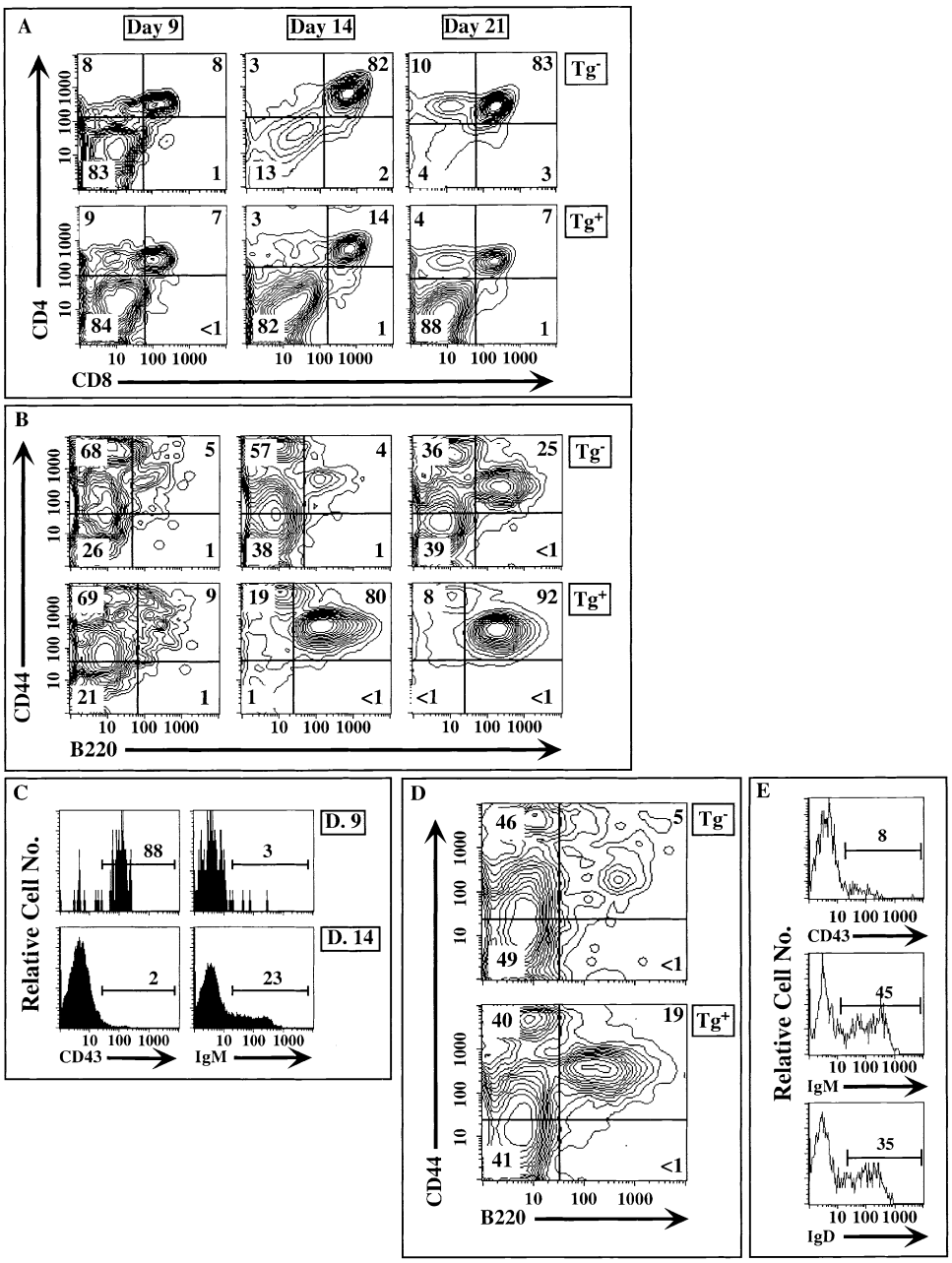

Figure 2. Ectopic Lunatic Fringe Inhibits T Cell Development and Augments B Cell Development from HSC and LP in the Thymus

(A) Decreased production of DP and SP thymocytes from LP injected into Tg^+ thymic lobes. The contour plots depict CD4 versus CD8 expression on $CD45.1^+$ thymocytes derived from Tg^- LP that developed in Tg^- versus Tg^+ hosts. T and B cell-depleted BM cells (2×10^6) were injected into each thymic lobe of seven Tg^+ mice (B6.CD45.2) and four Tg^- (B6.CD45.2) mice. Single-cell suspensions from individual thymic lobes were analyzed 9, 14, or 21 days later for expression of CD45.1, CD4, and CD8 by three-color flow cytometry. The percentage of DP thymocytes in each sample is indicated.

(B) LP preferentially adopt a B cell fate in the thymus of Tg^+ mice. Contour plots depict CD44 versus B220 expression on donor LP-derived ($CD45.1^+$) DN thymocytes from the mice shown in (A). The percentage of $CD44^+B220^+$ cells within the DN subset of each sample is indicated. The mean absolute number of donor-derived B cells per thymic lobe in Tg^- versus Tg^+ hosts were 10^4 versus 3×10^4 (day 9), 8×10^4 versus 6×10^5 (day 14), and 3×10^5 versus 8×10^6 (day 21).

(C) Developmental progression of LP-derived thymic B cells in Tg^+ hosts. Histograms depict expression of CD43 and IgM on donor-derived ($CD45.1^+$) $CD44^+B220^+$ DN thymocytes from the Tg^+ hosts shown in (B).

(D) Ectopic Lunatic Fringe promotes HSC to adopt a B cell fate in the thymus. Contour plots depict expression of CD44 versus B220 on donor-derived (CD45.1) DN thymocytes from representative individual Tg^+ versus Tg^- B6.CD45.2 thymic lobes 3 weeks after intrathymic injection of 10^4 B6.CD45.1 bone marrow-derived HSC (Lin^- $Sca\text{-}1^+$ $c\text{-}Kit^+$) per lobe.

(E) Histograms depict expression of CD43, IgM, and IgD on $CD44^+B220^+$ DN thymocytes from the Tg^+ host shown in (D). The expression of each marker was assessed on donor-derived $CD45.1^+$ cells using four-color flow cytometry to identify $CD44^+B220^+$ thymic B cells within the DN subset. The percentage of $CD44^+B220^+$ cells positive for each marker is shown.

Table 2. Etopic Lunatic Fringe Impairs T Cell Development and Promotes B Cell Development from LP Injected into the Thymus

	Total	Donor DP		B Cells (Donor DN)	
Thymic Lobe	% CD45.1	%	No. × 10^6	%	No. × 10^6
(A) CD45.2 Tg^-	98	86	41.8	22	0.3
	98	86	83.4	30	0.7
	95	88	101.7	11	0.2
	96	82	64.5	25	0.4
(B) CD45.2 Tg^+	35	37	4.5	86	5.7
	47	8	0.9	93	9.7
	31	40	2.1	83	2.1
	50	13	1.7	94	10.1
	56	8	0.9	96	8.4

Bone marrow LP from Tg^- B6.CD45.1 mice were injected into each thymic lobe (2 x 10^6/lobe) of sublethally irradiated Tg^+ B6.CD45.2 (CD45.2 Tg^+) and Tg^- B6.CD45.2 (CD45.2 Tg^-) host mice. Three weeks later, total or DN thymocytes were evaluated for expression of CD45.1, CD4, and CD8 or B220, respectively.

fold higher than the ratios of the input progenitor cells. These data strongly suggest that ectopic Lunatic Fringe inhibits Notch-1 activation in LP and thereby subverts the T/B lineage decision in the thymus.

LP Expressing Dominantly Active Notch-1 Are Insensitive to Modulation by Lunatic Fringe

In *Drosophila*, deletions of the extracellular and/or transmembrane regions constitutively activate Notch (Artavanis-Tsakonas et al., 1999) and render it insensitive to modulation by Fringe (Panin et al., 1997). Therefore, we tested whether constitutive activation of Notch-1 would prevent LP from choosing the B cell fate in response to ectopic Lunatic Fringe. LP from Tg^- B6.CD45.1 mice were infected with a retrovirus expressing murine Notch-IC and enhanced green fluorescent protein (GFP) as a bicistronic mRNA (pLZRS-Notch-IC). This Notch-IC protein promoted high-level expression of a HES-1 luciferase reporter gene in transient transfection experiments (data not shown), demonstrating that it effectively activates the Notch pathway. After infection with pLZRS-Notch-IC, LP were injected into Tg^+ thymic lobes. Three weeks later, the CD4/CD8 profile was similar among thymocytes that developed from Notch-IC-expressing ($CD45.1^+$ GFP^+) versus nonexpressing ($CD45.1^+$ GFP^-) LP in the same Tg^+ thymic lobe (Figure 4). In striking contrast, donor-derived B lineage cells were observed exclusively in the GFP^- thymocyte subset in all thymic lobes. These experiments demonstrate that LP-expressing activated Notch-1 are precluded from adopting a B cell fate in response to ectopic Lunatic Fringe.

Discussion

Notch-1 Activation Is Required to Suppress B Cell Development in the Thymus

In this study, we demonstrate that ectopic expression of Lunatic Fringe in cortical thymocytes prevents uncommitted LP from activating Notch-1, causing them to preferentially commit to the B cell rather than the T cell lineage (Figure 5). Moreover, we show that these B cells then progress through pro-B and pre-B cell stages to generate mature B cells in a very similar fashion to that seen in the bone marrow. Thus, our experiments have revealed that an important function of Notch-1 in T cell commitment is to suppress B cell development in the thymus. Similarly, Notch activation irreversibly inhibits neural crest stem cells from becoming neurons and allows them to adopt an alternate or secondary nonneuronal fate (Morrison et al., 2000). Because the neuronal fate is usually chosen in the absence of Notch signaling, it is often referred to as the primary or default fate. The primary role of Notch signaling is often to inhibit progenitors from adopting the default cell fate. Our data suggest that LP will choose the B cell fate by default in the thymus and that Notch-1 activation is required to inhibit B cell commitment and allow thymic LP to choose the secondary T cell fate.

Neither our data nor that of Pui et al. (1999) or Radtke et al. (1999) determine whether Notch-1 acts instructively or selectively to regulate the T/B lineage choice. In an instructive cell fate decision, Notch signals would induce a single progenitor to choose one cell fate over another. In a selective cell fate decision, Notch signals would regulate the survival and/or proliferation of progenitors that had already chosen a particular cell fate by another mechanism. Distinguishing between these possibilities is difficult and requires that a rigorous clonal analysis be performed to determine the frequency with which the progeny of single cells die. Using this type of assay, Morrison et al. (2000) have recently shown that Notch acts instructively to promote glial differentiation from neural crest stem cells. Given that clonal T/B progenitors exist (Kondo et al., 1997; Lacaud et al., 1998), it is tempting to speculate that Notch-1 acts instructively to induce T cell commitment. However, Notch-1 can inhibit apoptosis in committed T cell progenitors (Deftos et al., 1998), and it can induce apoptosis in B cells (Morimura et al., 2000); thus, a selective cell fate choice cannot be ruled out.

It is not yet clear how Notch-1 signaling regulates the T/B lineage decision at the molecular level, but we envision several possibilities. B cell commitment involves the activation of B lineage-specific gene expression by the bHLH transcription factors E2A and EBF, followed by the suppression of alternative lineage choices by Pax-5 (Busslinger et al., 2000). Pui et al. (1999) have shown that Notch-1 signals inhibit transcription induced by E2A but not EBF or Pax-5, suggesting that Notch-1 signals may inhibit the expression of B cell-specific genes. It is possible that Notch-1 signals

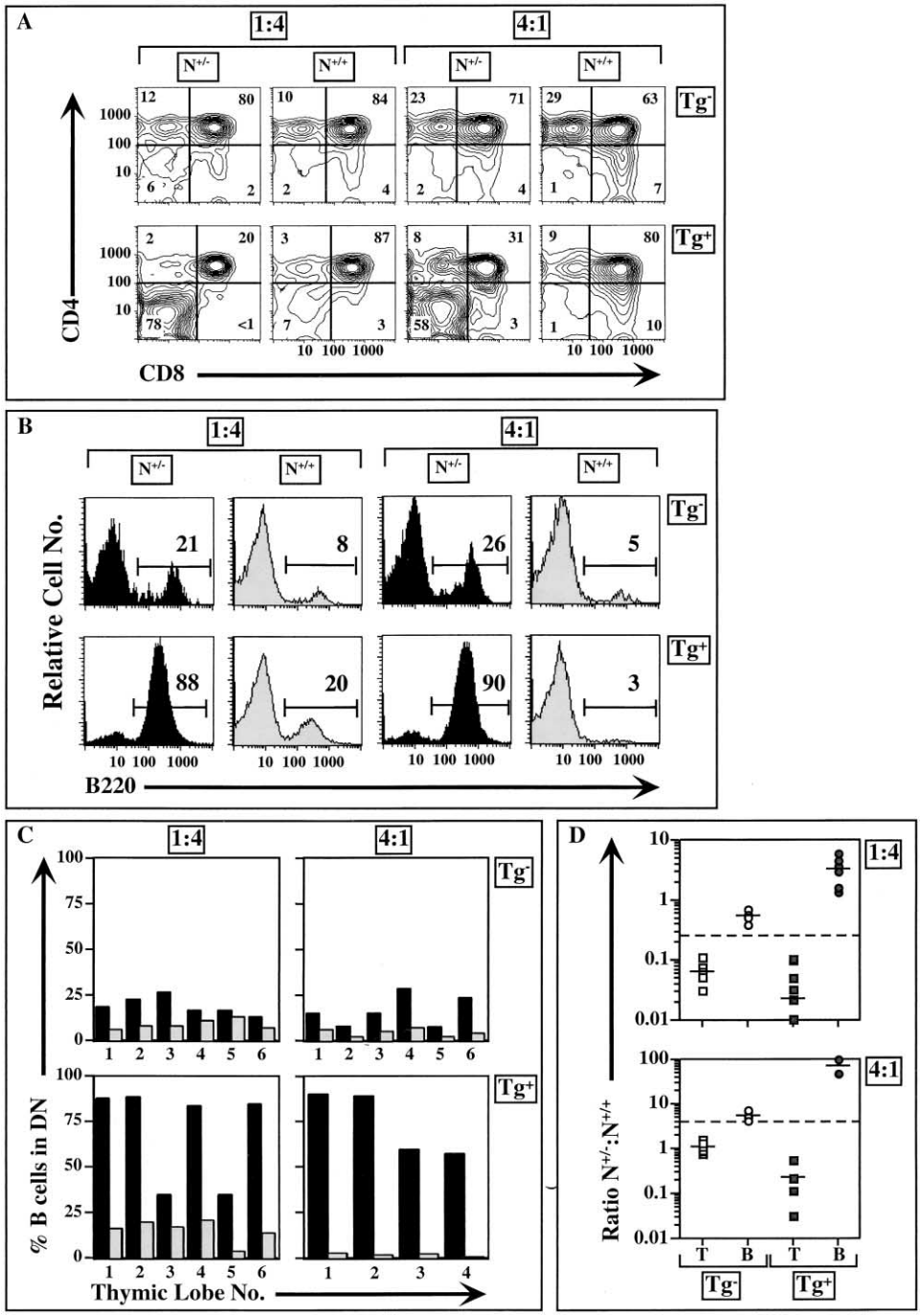

Figure 3. Reduced Notch-1 Gene Dosage Potentiates the Ability of Lunatic Fringe to Promote B Cell Development from Intrathymically Injected LP

Bone marrow LP from Notch-1$^{+/+}$ (B6.CD45.1) or Notch-1$^{+/-}$ (B6.CD45.1/CD45.2) mice were prepared as described in Figure 2A. Individual thymic lobes of sublethally irradiated (650 cGy) Tg^{+} or Tg^{-} host mice were injected with a 1:4 or 4:1 mixture of N1$^{+/-}$ and N1$^{+/+}$ LP (2×10^{6} total cells/lobe). The contribution of each LP donor to the T cell and B cell pool in each thymic lobe was analyzed 4 weeks later by four-color flow cytometry. Plots depict CD4 versus CD8 expression on total thymocytes (A) or B220 expression on DN thymocytes (B) derived from N1$^{+/-}$ and N1$^{+/+}$ LP within a single thymic lobe. (C) The percentage of B cells within the DN subset of each donor population is shown for all thymic lobes. (D) Ectopic Lunatic Fringe differentially affects the N1$^{+/-}$:N1$^{+/+}$ ratio in the T versus B cell lineages. Scatter plots show the ratio of N1$^{+/-}$:N1$^{+/+}$ cells within the T cell (squares) versus B cell (circles) lineage for individual Tg^{-} (white) or Tg^{+} (gray) thymic lobes. Dotted lines indicate the N1$^{+/-}$:N1$^{+/+}$ ratio of the input LP populations, and horizontal bars show the mean ratio for each group.

also positively regulate the expression of T cell-specific genes, because the pre-Tα gene has been identified as a potential Notch-1 target in immature thymocytes (Deftos et al., 2000). However, it is not known whether Notch-1 signaling is essential for the expression of pre-Tα or other T cell genes; thus, it remains to be deter-

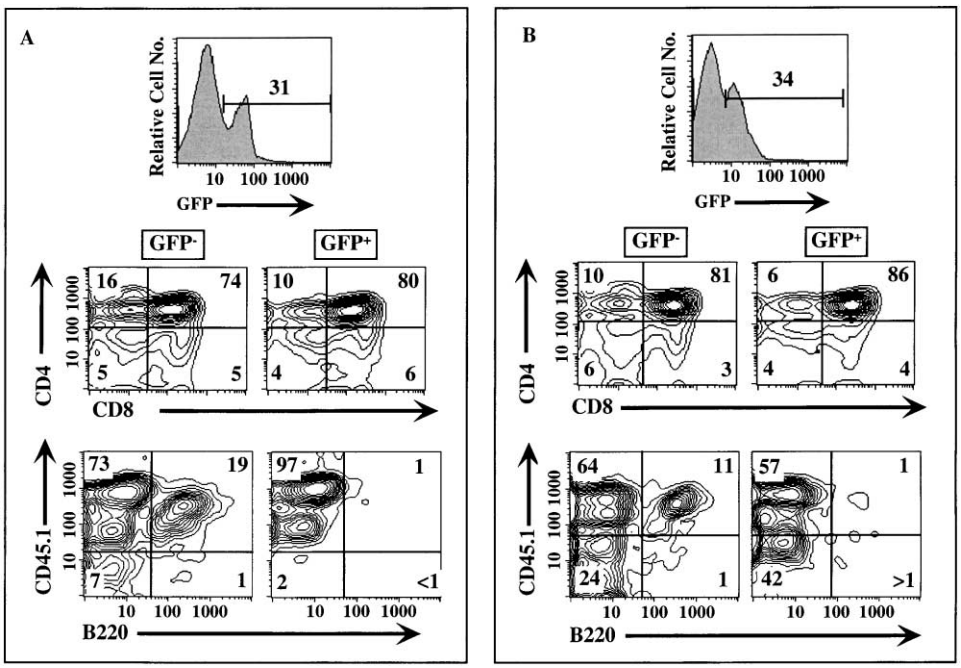

Figure 4. LP Expressing Constitutively Active Notch-1 Are Refractory to the Effect of Ectopic Lunatic Fringe

Prior to retroviral infection, LP were obtained by magnetically depleting bone marrow of Lin^+ cells (A) or by sorting Lin^- $Sca\text{-}1^+$ $c\text{-}Kit^+$ cells (B). Infected cells were injected into individual thymic lobes of sublethally irradiated Tg^+ B6.CD45.2 mice (Lin^- cells: 1.5×10^5/lobe; Lin^- $Sca\text{-}1^+$ $c\text{-}Kit^+$ cells: 3×10^4/lobe). Histograms depict GFP expression on donor-derived ($CD45.1^+$) thymocytes from representative Tg^+ thymic lobes 3 weeks later. The percentage of GFP^+ cells is indicated. Contour plots depict the expression of CD4 versus CD8 on GFP^- versus GFP^+ donor-derived ($CD45.1^+$) thymocytes (top) or CD45.1 versus B220 on GFP^- versus GFP^+ DN thymocytes (bottom). Four Tg^+ thymic lobes were analyzed in both experiments, and 94%–99% of total and all GFP^+ thymocytes were donor derived.

mined whether suppression of the B cell fate is the only function of Notch-1 in the T/B lineage choice.

Effects of Lunatic Fringe on Notch-1 Activity during Postcommitment Stages of T Cell Development

We showed that ectopic Lunatic Fringe decreases the numbers of T cell-committed DP and SP thymocytes that develop from LP in the thymus (Figure 2; data not shown). It is likely that the decrease in DP and SP thymocytes is due to the reduced generation of committed T cell progenitors rather than to a direct effect of ectopic Lunatic Fringe on these later stages of T cell development. Although several postcommitment aspects of T cell development can be influenced by ectopic Notch-1 activity (Robey et al., 1996; Washburn et al., 1997; Deftos et al., 1998, 2000; Kim and Siu, 1998), it has recently been shown that Notch-1 is not essential for these events (Wolfer et al., 2001). This finding accords with our observation that ectopic Lunatic Fringe inhibits Notch-1 activation in LP but does not inhibit the development of SP T cells from DP precursors. However, cortical thymocytes also express Notch-2, and Lunatic Fringe has been reported to enhance activation of Notch-2 in a cell culture model (Hicks et al., 2000). Given that the postcommitment aspects of T cell development occur normally in the presence of ectopic Lunatic Fringe (Figures 1 and 2), we think it unlikely that Lunatic Fringe-Notch-2 interactions are important in this context. Furthermore, we have not observed T cell tumors in a cohort of 1-year-old transgenic mice (data not shown), as would be expected if the Notch pathway was inappropriately activated. Collectively, these data argue that the sole effect of ectopic Lunatic Fringe in the thymus is to regulate Notch-1 activation in LP.

Lunatic Fringe Regulates Notch-1 Activation in LP

We have used two independent genetic approaches that together identify Lunatic Fringe as a potent upstream regulator of Notch-1 in LP that can subvert the T/B lineage choice in the thymus. The ability of ectopic Lunatic Fringe to induce a T/B cell fate switch in the thymus was greatly enhanced by a reduction in Notch-1 gene dosage in LP (Figure 3) and was ablated by constitutive activation of Notch-1 in LP (Figure 4). The preferential generation of B cells by $N1^{+/-}$ LP in Tg^- and Tg^+ thymic lobes occurred regardless of their abundance in the starting population. However, it is interesting to note that the potential of $N1^{+/+}$ LP to be inhibited by Lunatic Fringe was influenced by the frequency of $N1^{+/-}$ LP coinjected. Lunatic Fringe inhibited Notch-1 activation very efficiently in $N1^{+/+}$ LP when they were injected alone, since 70%–90% of their progeny were B lineage cells 3 weeks later (Figure 2). However, when $N1^{+/-}$ and $N1^{+/+}$ LP were coinjected at a ratio of 1:4, only 14%–20% of the $N1^{+/+}$ DN thymocytes were B cells (Figure 3C). Even fewer ($<5\%$) $N1^{+/+}$ LP became B cells when the starting ratio was 2:1 (data not shown) or 4:1, suggesting that when $N1^{+/-}$ LPs are in the majority, they somehow render $N1^{+/+}$ LP less sensitive to modulation by Lunatic Fringe. These observations suggest that the choice of

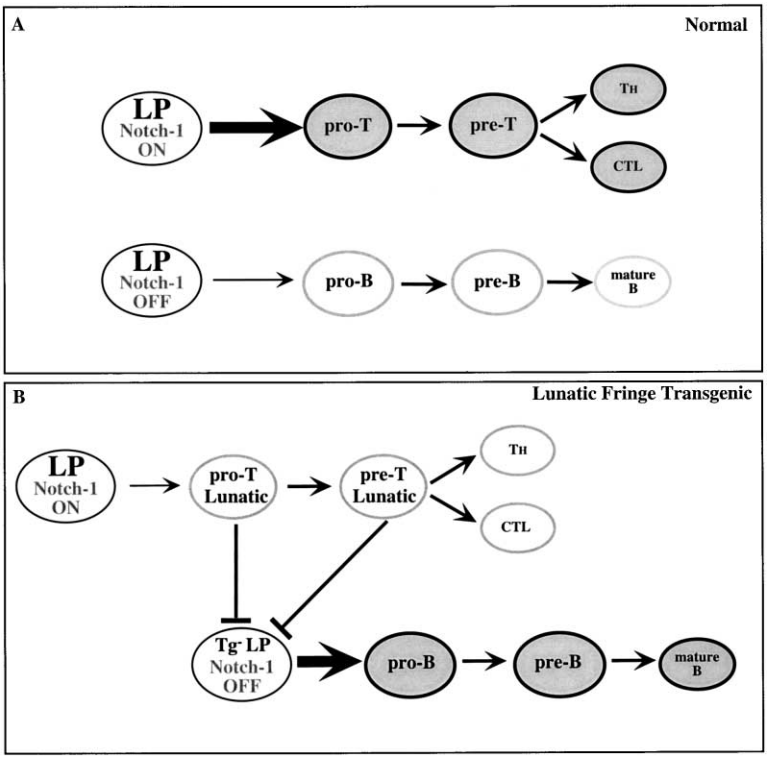

Figure 5. Nonautonomous Effect of Ectopic Lunatic Fringe on the T/B Lineage Choice in the Thymus

The schematic depicts the outcome of Notch-1 signaling and its effect on T/B cell fate in the normal (A) versus the Lunatic Fringe transgenic thymus (B). The major cell fate in each thymus is indicated by dark shading, whereas the minor cell fate is indicated by no shading. Based on the known developmental stage specificity of the Lck proximal promoter (Shimizu et al., 2001), transgenic Lunatic Fringe is predicted to be first expressed after T cell commitment at the pro-T cell stage. Data presented in Figures 2–4 show that Lunatic Fringe transgenic thymocytes act nonautonomously to inhibit Notch-1 activation in nontransgenic LP, causing them to adopt a B cell fate in the thymus (see text for further details).

T/B cell fate by a particular LP can be influenced by the degree of Notch-1 activity in neighboring cells, as has been shown for other Notch-regulated cell fate decisions (Artavanis-Tsakonas et al., 1999; Robey, 1999). One possibility is that Lunatic Fringe inhibits Notch-1 activation more rapidly in $N1^{+/-}$ than in $N1^{+/+}$ LP. Once inhibited, $N1^{+/-}$ LP may then promote Notch-1 activation in neighboring $N1^{+/+}$ LP, rendering the latter cells insensitive to Lunatic Fringe (Figure 4). While the molecular basis by which $N1^{+/-}$ and $N1^{+/+}$ LP communicate remains to be elucidated, our data suggest that there is a dynamic and competitive element to the way in which the Notch-1-Lunatic Fringe interaction regulates the T/B lineage decision in this system.

Our genetic data strongly argue that Lunatic Fringe ectopically expressed in immature T cells inhibits Notch-1 activation in LP. This finding is consistent with the activity of Lunatic Fringe in a cell culture model of Notch-1 activation (Hicks et al., 2000; Moloney et al., 2000). However, studies of Lunatic Fringe knockout mice (Evrard et al., 1998; Zhang and Gridley, 1998) have suggested that Lunatic Fringe positively regulates Notch-1 activation by two mammalian Delta homologs during somitogenesis (de Angelis et al., 1997; Kusumi et al., 1998). It is important to note that Fringe can inhibit or enhance Notch signaling, depending on which Notch ligand is involved. In developing fly wings, Fringe potentiates Notch activation by Delta but inhibits Notch activation by Serrate (Irvine and Wieschaus, 1994; Panin et al., 1997). Thus, Lunatic Fringe may have a positive or negative effect on Notch-1 activation depending on the developmental context and the Notch ligand. The fact that Lunatic Fringe negatively regulates Notch-1 in LP may reflect the involvement of a Serrate/Jagged ligand in the T/B lineage decision. However, the Notch-1 ligand(s) required for T cell commitment has not yet been identified.

Lunatic Fringe Nonautonomously Inhibits Notch-1 Activation in LP

Here we show that cortical thymocytes ectopically expressing Lunatic Fringe act nonautonomously to inhibit Notch-1 activation. This finding was unexpected in view of data identifying Fringe proteins as glysosyltransferases that modify the Notch receptor cell autonomously to modulate Notch signaling (Bruckner et al., 2000; Hicks et al., 2000; Moloney et al., 2000; Munro and Freeman, 2000). Nonetheless, Fringe and Lunatic Fringe can be secreted (Johnston et al., 1997; Panin et al., 1997), and secreted Lunatic Fringe was reported to induce mesoderm in vitro in frogs (Wu et al., 1996). Thus, secreted Lunatic Fringe could act nonautonomously to modulate Notch-1 activation in thymic LP, thereby affecting the T/B lineage decision. However, it is unlikely that the Fringe substrate UDP-*N*-acetylglucosamine would be found extracellularly. Therefore, we favor the alternative possibility that ectopic Lunatic Fringe alters the expression and/or function of Notch ligands (Panin et al., 1997; Artavanis-Tsakonas et al., 1999) in Tg^{+} thymocytes, causing them to inhibit Notch-1 activation in Tg^{-} LP. In accord with this notion, recent studies have shown that the *Drosophila* Notch ligands Delta and Serrate are both substrates for Fringe in vitro (K. Irvine, personal communication). The nonautonomy of Lunatic Fringe function in our system could thus reflect the direct modification of Notch ligands by Lunatic Fringe in vivo to regulate Notch-1 activation during the T/B lineage decision.

Implications

In the normal thymus, most LP choose the T cell lineage. Because T cell commitment is dependent on Notch-1 signaling, it is reasonable to speculate that the normal thymic microenvironment efficiently promotes Notch-1 activation in LP (Figure 5). However, a few LP choose the B cell fate in the normal thymus (Figure 2), likely because they fail to activate Notch-1 (Figure 5). We show here that ectopic expression of Lunatic Fringe in the thymus nonautonomously inhibits LP from activating Notch-1, causing them to develop into B cells (Figure 5). Thus, following the Notch-1-dependent choice of T/B cell fate, the thymus can support both the T and B cell developmental programs. Conversely, ectopic Notch-1 activation in LP causes T cell development in the bone marrow (Pui et al., 1999). An important implication of these findings is that the critical difference in the thymus versus bone marrow microenvironments, at least with respect to lymphocyte development, may be how efficiently they induce Notch-1 activation in LP. Our data identify Lunatic Fringe as a potent regulator of Notch-1 during the T/B lineage choice. While our studies do not identify the functions of endogenous Lunatic Fringe, they raise the possibility that Lunatic Fringe and/or its homologs may regulate Notch-1 activation in LP to ensure that T and B cells develop in different tissues.

Experimental Procedures

Mice

Wild-type B6.SJl-Ptprc^{a}/BoAiTac (B6.CD45.1; Taconic, Germantown, NY) between 6 to 10 weeks of age were used as bone marrow donors for purification of HSC and LP. B6.SJL-Ptprc^{a}BoCrTac-Rag2^{tm1} (RAG-2$^{-/-}$ B6.CD45.1, 10 weeks of age; Taconic) were used as bone marrow recipients. C57BL/6J-Notch1^{tm1Con} (N1$^{+/-}$ B6.CD45.2) were purchased from the Jackson Laboratory (Bar Harbour, ME) and were maintained by breeding to C57BL/6 Ka (B6.CD45.2) mice. N1$^{+/-}$ progeny were identified by polymerase chain reaction (PCR) amplification of tail DNA using neomycin-specific primers (primer sequences available on request). N1$^{+/-}$ (B6.CD45.1/CD45.2) F_1 mice were generated by crossing N1$^{+/+}$ B6.CD45.1 with N1$^{+/-}$ B6.CD45.2 mice. C57BL/6 Ka (Thy-1.1) and C57BL/6 Ka mice were bred and maintained at our own breeding colony.

Transgenic PrLFng mice were generated as follows: the full-length cDNA encoding murine Lunatic Fringe with a 3′ FLAG tag was cloned into the p1017 transgene vector downstream of the Lck proximal promoter and upstream of the 3′ UTR of human growth hormone gene (hGH). A Not1 fragment containing the Lck promoter Lunatic Fringe cDNA and 3′ UTR of hGH was purified and microinjected into (B6xSJL)F1 embryos. Transgenic offspring were identified by PCR amplification of tail DNA with primers specific for Lunatic Fringe and hGH (primer sequences available on request). All transgenic founders were confirmed by Southern blot analysis of tail DNA with a hGH probe. Three Tg^{+} founder lines (17, 19, and 20) were established, and transgene expression in thymocytes was confirmed by reverse transcription-PCR and Western blot analysis (data not shown). Each transgenic line was maintained by backcrossing (n = 7) Tg^{+} progeny to C57BL/6 Ka (Thy-1.1; B6.CD45.2) mice. All mice were housed under pathogen-free conditions.

Antibodies and Flow Cytometry

Thymocytes were stained with PE anti-CD4 (Pharmingen, Mississauga, Ontario, Canada) and APC anti-CD8α (Cedarlane, Hornby, Ontario, Canada), and immunoflourescence was quantified by two-color flow cytometry. DN thymocytes were identified by lack of staining with biotinylated antibodies specific for CD4, CD8, CD3ϵ, and TCRβ detected by Streptavidin Cy5-PE. DN thymocytes were evaluated for expression of CD44 (PE; Pharmingen) and CD25 (FITC; Pharmingen) or CD44 (PE) and B220 (APC; Cedarlane) by three-color flow cytometry. In Figure 2C, FITC-conjugated antibodies specific for CD43, IgM, IgD, or Igκ (all purchased from Pharmingen) and four-color flow cytometry were used to identify the maturational stage of B220^{+}/CD44^{+} DN thymocytes. Where required, antibodies directed against CD45.1 and/or CD45.2 (FITC or PE conjugated; Pharmingen) were included, and immunofluorescence was quantified by four-color flow cytometry. All other antibodies were purchased from Pharmingen.

Bone Marrow Chimeras and Intrathymic Injections

RAG-2$^{-/-}$ mice were lethally irradiated (1000 cGy) up to 4 hr prior to intravenous injection with 10×10^6 T cell-depleted bone marrow cells from a single donor (B6.CD45.1 Tg^{-} or B6.CD45.2 Tg^{+}) or with a mixture of 5×10^6 cells from each donor (B6.CD45.1 Tg^{-} and B6.CD45.2 Tg^{+}). Intrathymic injections were performed as previously described (Guidos et al., 1989). Host mice were 4 to 6 weeks of age and were sublethally irradiated (650 cGy) up to 3 hr prior to injection. Thymocytes from individual lobes were analyzed as indicated 9 to 21 days postintrathymic injection by flow cytometry.

Purification of HSC and LP

Bone marrow cells from Tg^{-} B6.CD45.1 mice were enriched for LP by magnetic bead depletion of T and B cells that express B220 or Thy1. HSC/LP were isolated and purified as follows: Bone marrow cells (B6.CD45.1) expressing markers of the myeloid (GR-1, Mac-1), lymphoid (B220, CD4, CD8, and CD3), and erythroid (TER-119) lineages were removed using sheep anti-rat IgG magnetic beads (Dynal Inc., Great Neck, NY) and purified antibodies directed against the lineage markers. Lin^{-} Sca-1^{+} c-Kit^{+} cells were then isolated (99% pure) by cell sorting and were injected into each thymic lobe (10^4/lobe) of Tg^{+} or Tg^{-} B6.CD45.2 mice.

Plasmids

The coding sequence for Notch-IC (amino acids 1750–2294) was excised from the full-length cDNA and was modified with linker sequences to add a Kozak sequence, AUG and FLAG tag to the 5′ end as previously described (Robey et al., 1996). This modified Notch-IC coding region was cloned into pcDNA3 and into the pLZRS retroviral vector, kindly provided by Dr. Garry Nolan (Stanford University, Stanford, CA). The Notch-IC protein was highly expressed following transient transfection of 293T cells with pcDNA3-Notch-IC, as shown by Western blot analysis with the anti-FLAG M2 antibody (Sigma, St. Louis, MO). This Notch-IC construct robustly activated the Notch signaling pathway, as pcDNA3-Notch-IC induced expression of a pGL3-HES-1 luciferase reporter gene 130-fold over background in NIH 3T3 cells. The murine HES-1 promoter region (−194 nt to +160 nt) was amplified and cloned into the pGL3 luciferase plasmid (Promega, Madison, WI) as described (Jarriault et al., 1995).

Retroviral Infections

Retrovirus was produced by transient transfection of the pLZRS-Notch-IC construct (10 μg) into the Phoenix ecotropic helper cell line (Garry Nolan, Stanford University) using calcium phosphate. Forty-eight hours later, retrovirus-containing supernatant was harvested and concentrated (10- to 50-fold) overnight by ultracentrifugation. The targets for retroviral infection were either bone marrow cells magnetically depleted of Lin^{+} cells or sorted Lin^{-} Sca-1^{+} c-Kit^{+} cells. In both cases, bone marrow cells were harvested from B6.CD45.1 mice 5 days after intravenous injection of 5-fluorouracil (150 mg/kg). LP were prestimulated for 2 days in complete Iscove's modified Dulbecco's medium supplemented with recombinant murine stem cell factor (100 ng/ml), recombinant human interleukin-11 (10 ng/ml) (both from Stem Cell Technologies, Vancouver, British Columbia, Canada), and recombinant human Flt-3/Flk-2 ligand (100 ng/ml) from R&D Systems (Minneapolis, MN). Cells were then infected with concentrated retrovirus by spinoculation (650 $\times$ g, 45 min) in the presence of polybrene (8 μg/ml), resuspended in media with the previously mentioned growth factor cocktail, and incubated for 2 hr at 32°C. The spinoculation was repeated, and cells were then cultured with growth factors for at least 2 hr prior to injection into individual thymic lobes of sublethally irradiated Tg^{+} B6.CD45.2 mice (Lin^{-} cells: 1.5×10^5/lobe; Lin^{-} Sca-1^{+} c-Kit^{+} cells: 3×10^4/lobe).

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